

Treatment of non-healing wounds with autologous bone marrow cells, platelets, fibrin glue and collagen matrix

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Abstract

Background aims. Recalcitrant diabetic wounds are not responsive to the most common treatments. Bone marrow-derived stem cell transplantation is used for the healing of chronic lower extremity wounds. **Methods.** We report on the treatment of eight patients with aggressive, refractory diabetic wounds. The marrow-derived cells were injected/applied topically into the wound along with platelets, fibrin glue and bone marrow-impregnated collagen matrix. **Results.** Four weeks after treatment, the wound was completely closed in three patients and significantly reduced in the remaining five patients. **Conclusions.** Our study suggests that the combination of the components mentioned can be used safely in order to synergize the effect of chronic wound healing.

Key Words: chronic wounds, diabetes, healing, stem cells

Introduction

Impaired local blood circulation as a result of micro- and macrovascular disease and peripheral neuropathy causes foot ulceration in up to 25% of patients with diabetes mellitus (DM) (1). Foot ulceration is associated with increased morbidity and mortality, has a negative impact on the quality of life of diabetic patients and poses a serious burden on the health care system (2,3). The cost of treating one diabetic foot ulcer has been estimated to be \$5000–\$8000 (1).

The management of diabetic foot ulcers is a major clinical challenge. Current therapy of diabetic foot ulcerations includes: (i) debridement; (ii) offloading and (iii) supplementary treatments. However, the response to treatment is often poor and the outcome disappointing. These wounds place a limb at risk of infection and amputation. Every year in the USA, 82 000 limb amputations are performed in patients with DM (4). Therefore there is an urgent need to investigate more effective supplementary treatments for diabetic foot ulcerations to a level that exceeds current standard care measures.

Considering the pathophysiology of chronic non-healing wounds, the more widely recognized causative factors are (i) phenotypically altered and/or senescent mesenchymal cells that fill the dermis of the skin (5–7); (ii) significantly decreased local concentration, stability and bioavailability of growth factors; (iii) a significantly higher local activity of matrix metalloproteinases that degrade the extracellular matrix, impair tissue repair and suppress cell proliferation and angiogenesis (8,9). Accordingly, the success and efficacy of chronic wound healing depends on the reconditioning of phenotypically altered resident cells, and correction of the wound matrix (10).

After wound debridement (which aims to convert the chronic wound to an acute wound), offloading and proper attention to the bacterial burden, the main contributing components in the process of chronic wound healing are mesenchymal stromal cells (MSC), growth factors and extracellular matrix. These components can promote angiogenesis, permanent matrix synthesis and re-epithelialization of the healing wound.

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(Received 14 August 2010; accepted 22 December 2010)

ISSN 1465-3249 print/ISSN 1477-2566 online © 2011 Informa Healthcare
DOI: 10.3109/14653249.2011.553594

Several novel approaches for diabetic foot ulceration treatment have been proposed recently. These suggest the use of bone marrow stem cells (11), platelet-derived wound healing factors (12), fibrin glue (13) or bone marrow-impregnated collagen matrix (14). Each of these approaches has been reported to increase the response time of healing chronic wounds. But, as the wound environment is dynamic and requires the presence of all 'contributing components', it is unlikely that one type of treatment alone can bring a wound to complete closure (15,16).

We report here on the first eight patients included in a larger clinical trial aiming to evaluate the treatment of DM patients with recalcitrant diabetic wounds using a combined application of four recently proposed approaches (bone marrow MSC, platelet growth factors, fibrin glue and bone marrow-impregnated collagen matrix). To the best of our knowledge, this is the first time that this combination has been applied to chronic wound treatment.

Methods

Eight patients with foot ulcers that did not respond to any conventional therapy were included. The patients' characteristics, past medical history, wound size and duration of wound are presented in Table I.

The study was conducted in accordance with the principles of the Declaration of Helsinki 1996 and good clinical practice standards. The study protocol, informed-consent form and other study-related documents were reviewed and approved by the Human Research Ethics Committee of Mashhad University of Medical Sciences (Mashhad, Iran). All patients were able to read and understand and were willing to sign the informed-consent form for the study.

Inclusion criteria were the presence of a non-healing diabetic wound for at least 3 months, age older than 18 years (both genders), and agreement of the patient to comply with the protocol requirements, including self-care of wounds and all

follow-up visit requirements. Exclusion criteria were pregnancy, active or previous (within 8 weeks of the study screening visit) chemotherapy, physical or mental disability (if not cared for, i.e. home nursing, etc.), current participation in another clinical investigation, a life expectancy of less than 6 months and limb-threatening complications or the need for immediate limb amputation. In addition we excluded patients with any of the following medical conditions: current candidates for vascular surgery, angioplasty or stenting, patients presenting with the clinical characteristics of cellulitis at the ulcer site, purulence or sinus tracts that could not be removed by debridement on the foot to be treated, malignant wounds, renal failure, vasculitis or connective tissue disease, bone marrow involvement (lymphoma-leukemia), average blood sugar > 200 mg/dL during hospitalization, patients treated with corticosteroids, lactating mothers and patients with any systemic infection.

Two days before bone marrow aspiration, 100 mL peripheral blood were taken and platelets and fibrin glue were prepared according to standard procedures (17,18). The platelets were prepared with a first centrifugation at 2000 *g* for 2 min and then a second centrifugation at 4000 *g* for 8 min, and the supernatant plasma was separated. The fibrinogen concentrate was prepared from the separated plasma using the cryoprecipitating method. Following a -70°C freeze and a 4°C thaw, citrate-phosphate-dextrose-adenine anticoagulant (CPD-A) plasma was centrifuged at 6500 *g* for 5 min. The supernatant plasma was removed to a final volume of 4 mL, mixed with platelets and stored at -30°C. Bone marrow (mean \pm SD, 207.875 \pm 78.5 mL, range 119–325 mL) was aspirated under epidural anesthesia from the ileum, into commercial 450-mL triple blood donation bags, containing 63 mL CPD-A in bag 1 and 100 mL saline-adenine-glucose mannitol (SAG-M) in bag 2. Bone marrow (BM)-total nucleated cells (TNC) (BM-TNC) were separated and concentrated to 95% purity and to a final volume of about 8 mL using

Table I. Patient characteristics, past medical history, wound size before and after treatment, duration of wound, volume of bone marrow aspiration and number of injected TNC.

	Age (years)/ gender	Duration of diabetes (years)	Duration of wound (months)	Wound area at treatment initiation, (cm ²)	Wound area after 4 weeks, (cm ²)	Volume of bone marrow aspiration (mL)	Number of TNC injected ($\times 10^8$)
1	48/M	20	36	21.6108	Closed	183	16.9
2	49/M	20	24	3.9395	Closed	135	18.8
3	57/M	20	3	3.5574	Closed	325	22.1
4	46/F	5	36	12.0923	2.4662	203	15.0
5	52/M	12	36	4.5633	3.5447	251	15.7
6	55/F	6	12	2.7058	1.211	119	11.4
7	66/M	3	12	2.2439	0.6979	305	22.8
8	55/F	20	24	10.5851	4.5139	142	19.43

F, female; M, male.

hydroxyethyl starch (HES) (19,20). After removal of aliquots for routine tests, HES (HAES-sterile 10%; Fresenius Kabi, Deutschland GmbH, Bad Homburg, Germany) was added to the bone marrow blood in the collection bag (bag 1) to obtain a final concentration of 2%. In addition, 26 mL HES were added to the bag that contained 100 mL SAG-M (bag 2). Bag 1 was hung on a stand for 45 min to allow red blood cell (RBC) sedimentation. The supernatant was slowly expressed using a plasma extractor into bag 3 (an empty bag) and, as soon as red cells started to enter the connecting tube, the connecting tube was clamped temporarily. In order to recover any remaining BM-TNC trapped between the sedimented RBC, the contents of bag 2 (100 mL SAG-M plus 26 mL HES) were transferred to bag 1, which contained the RBC. The connecting tube was temporarily clamped and bag 1 was shaken gently, hung for 45 min and the supernatant then transferred to bag 3. Bag 3 was centrifuged at 400 *g* for 12 min. After completion of centrifugation, the supernatant plasma was transferred back to bag 2 using a plasma extractor and the cells were resuspended in about 8 mL of the remaining plasma. The numbers of BM-TNC, mononuclear cells (MNC) and RBC were determined using an automated hematology analyzer (Sysmex Kx-21; Japan).

Prior to the application of BM-TNC, the area of necrotic and devitalized wound was debrided surgically until bleeding was recognized macroscopically. This allowed the bone marrow cells to come into contact with viable wound tissue. About 5 h after marrow aspiration, 5 mL of BM-TNC were implanted in the wound by 1.5-cm deep injections at various sites and the margin of the wound, using a 23-gauge needle.

Following the injection, 2 mL BM-TNC were mixed with platelets and fibrin glue, applied to the wound and allowed to form a clot on the wound (fibrin matrix acts as a provisional scaffold for cells). Collagen matrix (Surgicoll®; MBP, Medical Biomaterial Products, GmbH, Tehran, Germany) was then impregnated with 10 mL BM-TNC suspension (1 mL BM-TNC mixed with 9 mL serum) and placed on the fibrin clot. Finally, paraffin gauze pads were placed over the wound and a bolster of rolled gauze pads placed over the paraffin gauze. This dressing was then wrapped with rolled gauze. After 3 days, the entire dressing was removed and the wound irrigated with isotonic sodium chloride solution. The wound was then covered again with gauzes as described above, and each day the entire dressing was removed and irrigated with isotonic sodium chloride solution. The wound was closely observed for 4 weeks for the formation of granulation tissue and closure. After discharging, the patients were followed up regularly for ulcer recurrence and any other possible complications.

Results

The results are presented at Table I. The GraphPad Instant statistical package (GraphPad Software Inc.) was used for statistical analysis. The level of statistical significance was set to $P < 0.05$. The wounds of three patients were completely closed after 4 weeks and there was a significant wound reduction after treatment ($P < 0.05$) in the remaining patients. Data on the identification of various cell types before the process of BM-TNC separation and after the process for the final product are presented in Table II. Pictures of the wounds, before and after treatment, are presented in Figures 1–3 following the same numerical order as in Table I.

Discussion

We report here on eight patients with recalcitrant diabetic wounds that were treated using a combination of autologous bone marrow cells, platelets, fibrin glue and collagen matrix. The patients had not responded to traditional modalities of treatment, including debridement, offloading and complementary therapies (such as antibiotic therapy, blood glucose level control, administration of zinc sulfate and multivitamins, and irrigation of wound with normal saline and dressings). However, after treatment with the combination described, three of the eight wounds were completely healed while the rest had improved significantly.

Wound healing is the sum of interrelated events, the common aim of which is the restoration of injured tissue. Optimum healing of a wound requires a well-orchestrated integration of the complex biologic and molecular events of cell migration, proliferation, extracellular matrix deposition and remodeling (21).

In the present study we used bone marrow cells, concentrated in a small volume, using a recently described technique able to remove most of the RBC from bone marrow aspirate, thus achieving small volumes useful for cell therapy (19). The bone marrow is an important source of hematopoietic stem cells, which regularly regenerate components of blood, and non-hematopoietic stem cells, including MSC which can be differentiated into several other cell types such as vascular endothelia, neurons,

Table II. Data on the identification of various cell types before the process of BM-TNC separation and after the process of the final product.

Cell type	TNC ($\times 10^8$)	MNC ($\times 10^8$)	RBC ($\times 10^9$)
Pre-process	30.2 \pm 6.9	10.3 \pm 3.0	229.2 \pm 62.5
Post-process	28.4 \pm 5.9	9.8 \pm 3.6	9.8 \pm 3.5
% yield	94.1 \pm 3.1	95.3 \pm 5.32	95.6 \pm 1.2 ¹

¹RBC depletion, %. Data are presented as mean \pm SD.



Figure 1. (A) Prior to treatment and before wound debridement in patient 1. (B) Appearance immediately after debridement and prior to application of TNC. (C) Appearance after 2 weeks of treatment. (D) Appearance after 4 weeks of treatment.

fibroblasts and skin keratinocytes. Considering the plasticity of bone marrow stem cells to produce new skin cells, it is conceivable that they may replenish lost cells during wound healing (22,23). Therefore, these cells are recognized as key players in tissue regeneration and, under appropriate conditions, it is considered that these cells can rejuvenate or rebuild tissue compartments (24). Several studies suggest that bone marrow-derived stem cells may contribute to wound repair, either by self-proliferation and differentiation or by releasing

regulatory cytokines. It has been proposed that stem/progenitor cells may be mobilized to leave the bone marrow, home to injured tissues and participate in repair and regeneration (25). Direct injection of bone marrow-derived MSC or endothelial progenitor cells into injured tissues shows improved repair through mechanisms of differentiation and/or release of paracrine factors (26).

Valbonesi *et al.* (27) promoted skin wound/lesion repair in two patients by using fibrin-platelet glue combined with HLA-compatible (two mismatches)

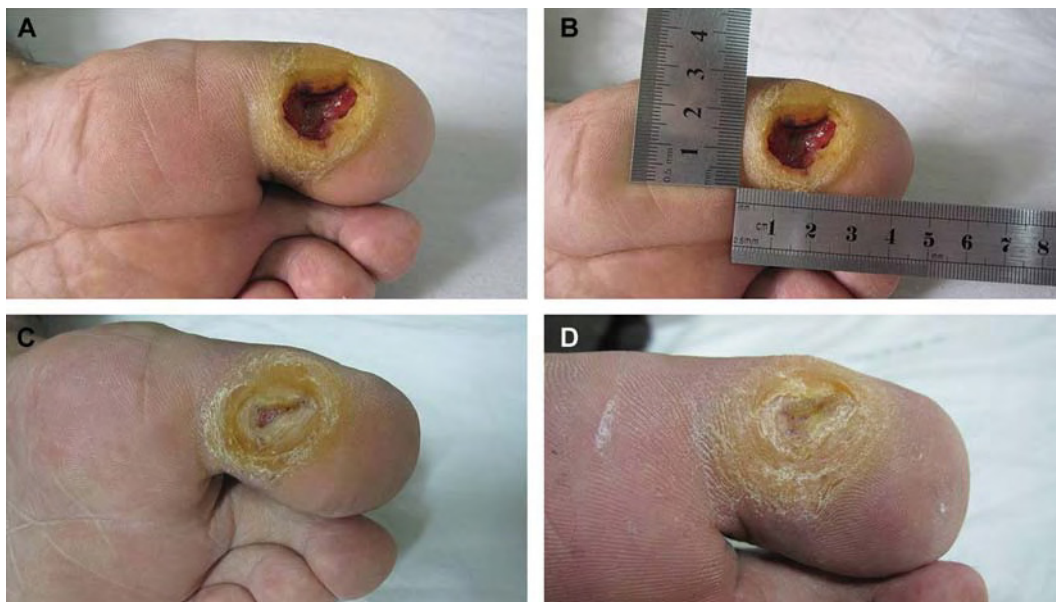


Figure 2. (A) Prior to treatment and before wound debridement in patient 2. (B) Appearance immediately after debridement and prior to application of TNC. (C) Appearance after 2 weeks of treatment. (D) Appearance after 4 weeks of treatment.



Figure 3. (A) Prior to treatment and before wound debridement in patient 3. (B) Appearance immediately after debridement and prior to application of TNC. (C) Appearance after 2 weeks of treatment. (D) Appearance after 4 weeks of treatment.

buffy coats containing allogeneic CD34⁺ cord blood cells; no graft versus tissue reaction was seen in patients with a follow-up of 3–7 months. Badiavas & Falanga (11) and Badiavas *et al.* (28) treated chronic wounds by applying autologous bone marrow aspirate and cultured bone marrow stem cells directly (11,28). Rogers *et al.* (15) applied/injected bone marrow aspirate containing marrow-derived cells locally into complex lower extremity chronic wounds of different etiologies (three cases) and suggested that bone marrow aspirate, applied topically and injected into the wound periphery, may be a useful and potentially safe adjunct to wound simplification and ultimate closure. Other studies have also shown the benefit of using bone marrow cells to heal difficult wounds (29,30). It should be noted that, in the present study, significantly higher amounts of cells (RBC-replenished bone marrow cell concentrate) were used at a single injection, as higher amounts of aspirated bone marrow blood were collected in comparison with Badiavas *et al.* (aspirated bone marrow blood 10–25 mL) (28). We were also not able to compare the dose–effect of injected TNC with other studies, which is related to the type of cells injected into wound. Badiavas *et al.* (28) injected fresh bone marrow blood (2–4 mL) and cultured bone marrow blood (MSC $4.5\text{--}5.2 \times 10^6$) and, to the best of our knowledge, a bone marrow cell concentrate injection for the treatment of chronic wounds has not hitherto been reported.

In this study, because the patients had different categories of wounds [deep wounds, undermined wounds (three cases) and tunneling wounds (three

cases), and superficial wounds (two cases)], the dose–effect of TNC on wound size closure could not be calculated statistically.

In order to increase cell concentrations, Falanga (24) delivered cultured autologous bone marrow-derived MSC in a fibrin spray into murine and human cutaneous wounds, and observed an accelerated healing. Nakagawa *et al.* (31) reported treatment of non-healing ulcers by applying MSC together with fibroblast growth factor (FGF) in an experimental animal skin defect model. It is known that cytokines, especially growth factors [transforming growth factor- β (TGF- β), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)], show significantly reduced expression in chronic wounds compared with acute wounds (32). There have been extensive investigations into wound healing after exogenous application of various growth factors (33). Accordingly we have combined stem cell application with platelets. Platelets have been used as a source for cytokines.

It is known that platelets can release a number of cytokines, such as PDGF, TGF- β , vascular endothelial growth factor (VEGF), EGF, insulin-like growth factor (IGF) and bFGF (34). There are several reports supporting the fact that these cytokines are critical to wound healing (35). Other investigators have used platelet growth factors to treat unhealing wounds (12). These cytokines play major roles in local inflammation, re-epithelialization, granulation tissue formation, neovascularization and extracellular matrix production (36). It should also be noted that

recombinant PDGF-BB isoform and human recombinant bFGF (separately) are Federal and Drug Administration (FDA) approved for the treatment of chronic ulcers (32).

Fibrinogen is converted to fibrin, which forms a cohesive network and provides an important temporary extracellular matrix for wound healing. Therefore we used fibrin glue to apply an admixture of platelets and stem cells to the wound. The structural composition of fibrin and the binding of fibrin to cells and proteins determines the wound healing process. Beyond behaving as a provisional matrix, fibrin actively recruits cells to trigger fibrin-mediated responses, such as cell adhesion, migration, proliferation and tubule formation. Fibrinogen promotes cell growth and vessel formation, which are beneficial during wound repair. This represents an ideal delivery vehicle for extra cells for the treatment of chronic wounds (13). Collagen matrix acts as a scaffold for regeneration, when applied to a tissue defect, sprouting capillaries, and fibroblasts migrate into the collagen, resulting in induction of angiogenesis and fibroplasia. Its efficacy has been demonstrated for the treatment of deep sacral ulcers (37). Therefore we used a bone marrow stem cell-impregnated collagen matrix to cover the wounds. It has been reported that this material significantly promotes the repair process, especially in early stages, and is used clinically by plastic surgeons in Japan for the treatment of chronic and acute wounds as a scaffold biomaterial (38–40).

In the pharmaceutical market, several cell-derived wound-care products are available. However, these products significantly increase the already high cost of diabetic foot treatment. Examples of these products include a bilayered living human skin equivalent, a human fibroblast-derived dermal substitute, and a human platelet-derived growth factor (41). A main benefit of the treatment presented here is that the procedure, because it is autologous, is very cost-effective compared with current commercial wound care products. One drawback of the method may be the need for bone marrow aspiration. However, this procedure, although invasive, causes only minimal discomfort to the patient that is outweighed by the beneficial effect of the therapy.

In spite of the many developing treatments for non-healing wounds, some wounds remain recalcitrant. We have presented the cases of eight patients with non-healing diabetic ulcers that were treated successfully with a combination of bone marrow stem cells, platelets, fibrin glue and collagen matrix. There was no evidence of local or systemic complications related to the procedure. On the basis of these results, we are running a larger study with the

aim of decreasing the need for amputation in chronic wound limbs.

Acknowledgments

The authors thank Biohellenika SA Biotechnology Company and Khorasan Razavi Blood Transfusion Center for providing the consumables, and Medical Sciences of Mashhad University for providing the facilities that enabled completion of this study.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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